FINAL REPORT

Preparation Of Guinea Pig Macrophage

FOR

Electrophoretic Experiments In Space

Grant No. ~~NSG-00008064~~

NSG-8062
NSG-8064

Submitted to

George C. Marshall Space Flight Center

cf the

National Aeronautics and Space Administration

May 1979

ACKNOWLEDGEMENTS

Alabama A&M appreciates Drs. Robert Synder and Linda Washington, as well as Adam Smoldgrad for their cooperation in the use of their laboratory facilities and expertise. We are especially grateful to Dr. Robert Allen abd Mr. Marion Kent for their support of this work.

ABSTRACT

The purpose of this study was to investigate methods of storage and cultivation of macrophage cells in preparation for space experiments.

Induction and Isolation of Macrophages; the animals used in this experiment were injected with 20 ml of mineral oil or 4% Thioglycolate into the peritoneal tissue. After four days the macrophage cells were harvested and washed with Hank's Balance Solution (HBSS).

Viabilities and Mobilities; the viabilities of macrophage cells were determined by adding one ml of cell suspension to 0.1 ml of trypan blue dye. The solution was mixed well and a drop was added to hemacytometer. The mobilities were determined by using a Particle Micro-Electrophoresis Apparatus (Preece and Light, 1975).

In the freezing method, 5 ml of macrophage cells were suspended in Medium 199 in a 15 ml ampule vial. Cryogenic protective agents (Glycerol and DMSO) were added at various concentrations. The cells were frozen at $-80^{\circ}C$ in a Kelvinator 100 freezer. At the end of 5 days, the viabilities and mobilities were determined.

Results showed that freezing and thawing immediately after extraction did not cause a change in viability or electrophoretic mobility of the macrophage cells. However, prolonged storage at $-80^{\circ}C$ did cause cell damage as indicated by a 95% reduction in viable cells.

Cell damage was decreased when Glycerol or Dimethyl Sulfoxide (DMSO) was added as a cryogenic protective agent. A 100% viability was observed

in cultivation experiments after two weeks due to the additional serum. Results from gamma-glutamyl transpeptidase study showed a zero activity rate.

It is suggested that a flat stationary field be used for the collection and use of macrophage. It was found that a 24-hour delay in obtaining macrophage cells helps to maintain a pure culture.

SUMMARY AND CONCLUSION

This paper describes further confirmation of the successful application of the Macrophage Electrophoretic Mobility (MEM) Test to the detection of malignant disease. MEM Test reported slower mobility rates for cells cultured in batch cultures and cells which were harvested from animals with tumors.

Experiments using gamma-glutamyl transpeptidase as a malignant tumor indicator showed no evidence. No appreciable amount of enzyme was produced in the presence of Walker 256 carcinoma or mammary tumor.

Data on the storage of guinea pig macrophage cells showed that freezing and thawing immediatedly after extraction did not cause a change in the viability, only after long term storage. Cultivation studies have also showed that by adding proper nutrients and changing of media every 12-hour period could maintain cell viability of 100% over a two-week period.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

The primary emphasis of immunological research in neoplasia during the past two decades has been the definition and characterization of tumor components that are absent from corresponding normal tissue. The existence of tumor-associated antigens on certain experimental neoplasm is now widely substantiated, and for some neoplasm these extend not only to those evoking transplantation resistance, but also to more widely cross-reacting entities of viral and embryonic type. With the increased interest in immunological research, this study presents methods of detecting malignant diseases by employing the specificity in most immune response. The technique involved is the Macrophage Electrophoretic Mobility (MEM) Test. The MEM Test is based on the net electronegative surface charge of guinea pig peritoneal macrophage as an indicator of lymphocyte antigen interaction. The data from the MEM Test indicated that a marked decrease of the net electrostatic charge (Field and Caspary, 1970) of peritoneal macrophages from tuberculin sensitivity in guinea pigs. When examining human macrophages it appears that the vast majority of patients with malignancies excluding those of the lymphoid system are sensitized to an antigen common to all cancers, irrespective of anatomical derivation or histological classification.

Studies of macrophage cells from unstimulated animals have revealed extensive changes during maturation in the morphology and in the content of some intracellular enzymes such as acid phosphates (Glenner et al, 1972). However, biochemical markers for the plasma membrane which reflect serological or enzymological differences in tumor macrophages will be investigated.

LITERATURE REVIEW

I. Physiological Functions of Macrophage Cells

Macrophage play many significant roles in the inflammatory pro-
cesses. They can control the production of humoral factors; they also
play a role in tumor cytotoxicity in the reticuloendothelial sys-
tem as well as in the inflammatory response occurring in a healing wound
(Wachsmuth and Stoye, 1977). The macrophage cell is one of the first
cell types to be established in primary culture and has been proven to
be useful in Cell Biology. Macrophages can be obtained from several
mammalian species. They have numerous functions such as: (1) scavengers
(clearing the body of dead cells, inorganic crystals and organic debris),
(2) cell-mediated defense system against certain types of microorganisms,
particularly falcultative and obligate parasites, (3) being involved in
antigen presentation to certain lymphocyte subpopulations, and finally,
they are thought to be involved in (4) in vivo regulation of granulocyte
proliferation and differentiation in vitro. To accomplish these func-
tions, marcophage has certain well-developed characteristics such as:
(1) highly developed phagocytic and pinocytic activity, (2) the ability
to adhere to a charged surface related to phagocytic ability, (3) the
ability to respond to certain classes of immunoglobulin G molecules and
for certain complement components, and (4) a sell developed arsenal
lysozomal (granule), acid hydrolase, oxygenase and catonic proteins.

II. Macrophage Electrophoretic Mobility: A Cancer Detector

Field and Caspary (1970) found that the electrophoretic mobility

rate of macrophage cells obtained from human subjects containing tumors are slower than normal human macrophages. Rawlins, Woods and Bagshawe (1975) took lymphocytes from a total of 161 subjects, including normal controls and patients with malignant and non-malignant conditions and have been investigated for their response to myelin basic protein, using the Macrophage Electrophoretic Mobility (MEM) Test. They confirmed that there was a high level of association between clinically evident cancer and a positive response. Lymphocytes from 24 to 45 patients with non-malignant inflammatory and ischaemic diseases also gave positive responses. In 46 patients with breast lumps studied before mastectomy or biopsy, the test was positive in 15 of 19 cases which proved to be malignant and in 5 of 27 which proved t be benign on histological examination. In the present form, the test is not sufficiently reliable for the diagnosis of early cancer. The results suggest that tissue necrosis in malignant and non-malignant conditions may be one of the factors resulting in sensitization to antigenic determinants present in preparation of myelin basic protein.

The Macrophage Electrophoretic Mobility Test was first suggested as being useful in the diagnosis of human malignant disease by Field and Caspary (1970) and Multiple Sclerosis (MS) by Field et al., (1974). These reports encouraged interest in defining the underlying mechanisms of growth. The test is similar in principle, to the better understood Macrophage Inhibition Factor (MIF) assay in which sensitized lymphocytes are stimulated by the appropriate antigen to release a factor inhibiting macrophage migration. A similar immunological basis for the two tests is suggested by

their capacity to detect lymphocyte sensitization to tumor basic protein in cancer patients (Hughes and Paty. 1971; Light et al., 1975). However, the MEM Test is credited with greater sensitivity.

The MEM Test has demonstrated sensitization to a variety of antigens including the purified protein derivative of the tuberculin in bacillus-fractioned thyroblogulin in Graves' Disease (Caspary et al., 1970), saline muscle and peripheral human nerve extract in myaethenia gravis (Field, 1973), encephalitogenic factor, and human sciatic nerve basic protein in neurological disease (Field et al.,1973). However, despite the apparent specificity of these responses, tumor basic protein will give a positive result of the MEM Test with lymphocytes from patients suffering from demyelinating disease, and myelin basic protein will evoke response with lymphocytes from patients with malignant disease.

The demonstration of cellular immune responsiveness to specific antigens in a variety of clinicopathological disease state has followed rapidly upon the development of quantitative and reproductible methods for the detection of lymphocyte sensitization. One such technique (Diengdol and Turk, 1968) demonstrated a marked decrease in the electrostatic charge of peritoneal macrophages from tuberculin-sensitive guinea pigs after challenged with tuberculin. They found that the effect was immunologically specific but did not appear to be dependent on the reaction of antigen and on circulating the reaction of antigen and circulating antibody occurring on the cell surface. The Macrophage Electrophoretic Mobility (MEM) Test of Field and Caspary (1970) was based on these observations, and was claimed to demonstrate changes in the net

electronegative surface charge of guinea-pig peritoneal macrophages following incubation with culture media containing lymphocytes and a previously encountered antigen. The variety of antigens reported to have been studied in the system is still very limited; however, particular interest arose from the report of Field and Caspary (1970) that lymphocytes from cancer patients respond to a his one-like substance extractable from the human brain and known as encephalitogenic factor (EF), a myelin protein (MBP) preparation. Later reports (Caspary et al., 1971) have described similar or more marked results, using the "cancer basic protein" (CaBP), an extract obtained by analogous procedure from a variety of human tumors.

III. Macrophage Membrane Bond Enzymes

Biochemical markers have been useful in the determination of malignant tumors. Histochemical analysis and centrifugal fractionation of tissue have shown that transpeptidase is a membrane-bound enzyme. Glenner et al., (1972) demonstrated by a specific histochemical method, located this enzyme on the brush border of the proximal convoluted tubules and loops of Henle in the kidney. The pattern of distribution has suggested the possibility that the enzyme may play a role in kidney function for example, in the tubular reabsorption of amino acids. Histochemical observations on the JuJunal mucosa of man which in some respects parallel those of Glenner, Folk and McMillan (1962) on kidney, have stimulated speculation regarding a general role for the enzyme in amino acid transport.

Szewczuk (1964) has shown that the tissue of man contained, in addition

to the membrane-bound enzyme, a small amount of gamma-glutamyl trans-peptidase which is recovered in the high speed supernatant after cen-trifugal fractionation of homogenates. Evidence was presented that the serum enzyme was closely related in properties to the soluble enzyme of liver.

MATERIALS AND METHODS

I.   Reagents

TC minimal essential medium eagle (MEM), TC 199 and TC Fetal Calf Bovine Serum (FCBS) desicated were obtained from Difco, Detroit, Michigan. Thioglycollate medium was purchased from Sigma Chemical Company, St. Louis, Missouri. Hank's Balanced Salt Solution (HBSS) was obtained from Flow Laboratories, Rockville, Maryland. Sodium Chloride came from Fisher Scientific Company, Fairlawn, New Jersey. Ten thousand units of Penicillin and 10,000 mcg Streptomycin/ml prepared in normal saline came from ICI Biological, Cary, Illinois. The uniform 10% solid polystyrene was purchased from Dow Chemical Company, Indianapolis, Indiana. All other chemicals were of analytical grade.

II.   Animals

Camm-Hartley strain guinea pigs were supplied by Camm Research, New Jersey and Sprague Dawley rats were obtained from Charles River Breeding Laboratories, Wilmington, Massachusetts. Rats infected with Walkers 256 Carcinoma, originally a gift from Dr. William R. Shapiro, Memorial Sloan-Kettering Cancer Center, New York, New York.

III.   Induction and Isolation of Macrophage for Freezing Studies

Normal guinea pig macrophages were produced by an intraperitoneal injection of 20 ml of sterile, warm, light mineral oil into Camm-Hartley strain guinea pigs of either sex, weighing in excess of 300 grams. No special precautions were taken to avoid infection, but only healthy-appearing guinea pigs were used. A heart puncture was performed four

days after the injections to remove blood serum. One hundred fifty ml of HBSS was then injected into the peritoneal cavity to provide an isotonic environment for the cells. A small incision was then made into the peritoneal cavity and the HBSS; a mineral oil solution containing macrophage cells was then removed. The cells were then centrifuged and washed with Media 199.

IV.   Induction and Isolation of Macrophage for Culture and Enzyme Assay

Guinea pig macrophages were obtained from peritoneal exudate essentially as described by Pritchard et al., (1973). Guinea pig macrophages were produced by two consecutive intraperitoneal injections with 10 ml of Thioglycollate broth, 24 hours apart. A washout was followed after 48 to 72 hours with the exudate and 150 ml of heparinized HBSS.

The suspension was centrifuged at 10,000 rpm for 5 minutes and resuspended in 20 ml of HBSS. The cells were added into a tissue culture flask and incubated for 2 hours at 70°C. The culture media was removed and washed twice with HBSS by very violent mixing to remove the unattached eryothrocyte cells. The macrophage cells were removed by using rubbing abrasive with a rubber policeman over the surface of the flask.

V.   Viabilities

Cell survival or viabilities were determined by dye exclusion (two minutes in 0.1 percent trypan blue), and a differential count was performed from a wet preparation stained with crystal violet (Weiler and Weiler, 1965). The percentage of viability was calculated by using the

following formula:

$$\% \text{ Viability} = \frac{\text{Non-Stained Cells X 100}}{\text{Total Cells}}$$

VI. Determination of Electrophoresis

Standard aseptic techniques were observed throughout the experiments. The NaCl buffer had minimal exposure to air and all transfers were done with sterile syringes. A particle Microelectrophoresis Apparatus (manufactured by Rank Brothers, Bottishma, Cambridge, England) was used for measuring the electrophoretic mobility (Figure 1).

Mobility measurements were made first by turning on the voltage and waiting for a well-focused cell. Movement of the cell was then observed from one graticule line across one or more graticule divisions to another graticule line. The process was repeated until a set of 10 readings was obtained. Calculations were done by dividing the cell per unit electrical field strength (X) into the electrophoretic velocity (V). Electrophoretic mobility is represented by U.

$$\frac{V}{X} = U$$

VII. Solid Tumor Transplantation

Two healthy rats bearing 14-day-old Walker tumors were sacrificed. The largest, most firm nonukerated tumor was then selected. The rat was then placed under a sterile laminar flora hood. The ventral area was then placed in cotton, soaked with 95% alcohol after which the tumor was removed. Only the non-necrotic and non-hemorrhage area of tumor (Pearl-White area taken generally at periphery) were cut into small
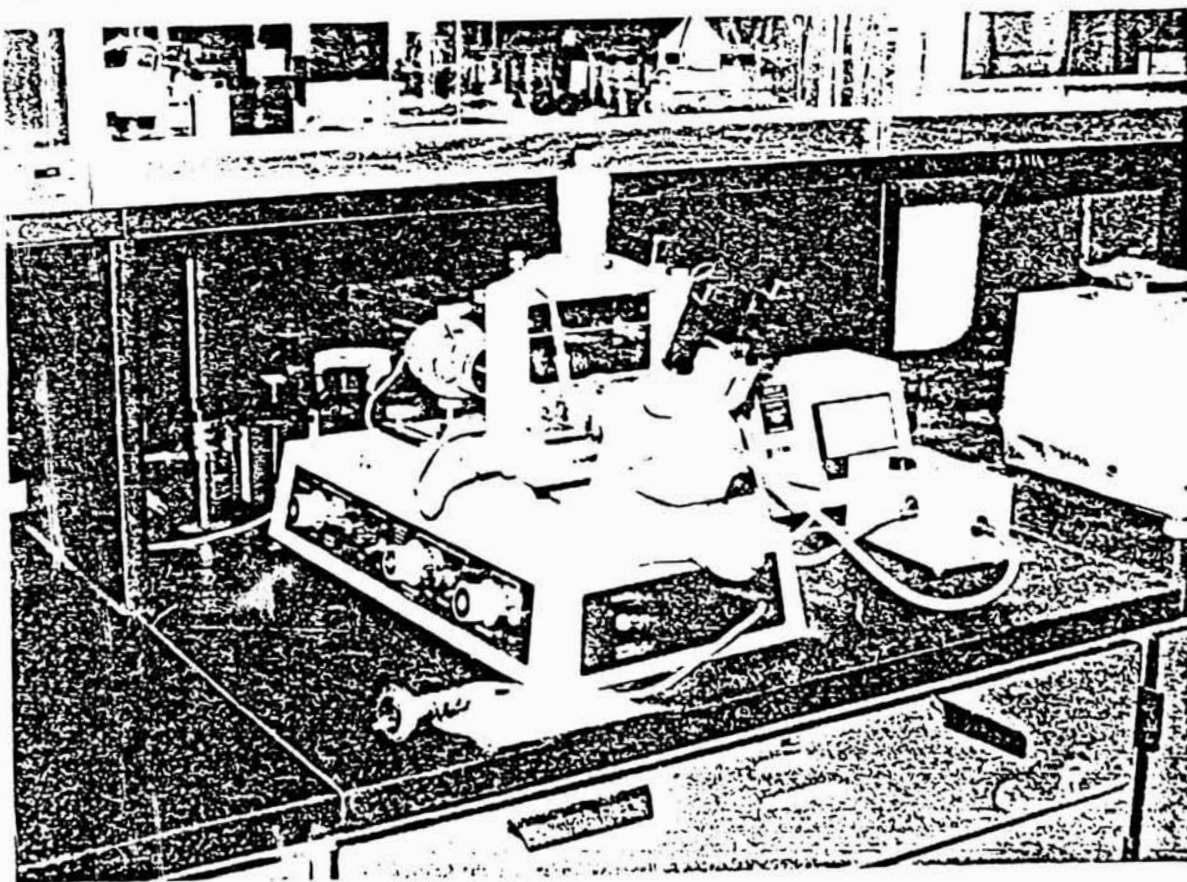
Figure 1. Rank particle Electrophoresis

cubes, approximately 8 mg pieces measuring approximately 2.5 mm. An incision was made into the muscles of the femor of ten rats using a sharp scapel. A fragment of the tumor was then placed into the muscle. The gamma-glutamyl transpeptidase enzyme activity was then measured on ten animals after 14 days. Mammary tumors were transferred by 10 mi injections into the right Aductor brevis.

VIII. Macrophage Culture System

A batch culture system was used to cultivate peritoneal macrophage cells. Peritoneal macrophage cells from guinea pigs were obtained essentially as described by Pritchard (1973). After the macrophages were obtained and resuspended in 100 ml of MEM containing 20% FCBS, 10,000 units penicillin and 10,000 mcg strtptomycin per ml, 25 ml of cell suspension was added to 14 Falcon Tissue culture dishes. The media in the flask was changed every 24 hours. Viabilities were then performed on to flasks every 48 hours.

IX. Freezing Methods

In 15 ml ampule vials, 5 ml of macrophage cells were suspended in TC 199 Medium. Cryogenic protective agents (glycerol and DMSO) were added at 5, 10, and 15 percent concentrations. The cells were consequently frozen at $-80^{\circ}C$ in a Kelvinator freezer, at a freezing rate of decreased temperature at $1^{\circ}C/5$ minutes. At the end of the fifth, 10th and 15th days, the viabilities and mobilities were determined.

X. Determination of Phagocytic Activity

A sterile pipet of one ml macrophage cell suspension was extracted

and added to a small petri dish containing a coverslip and 9 ml of TC 199 medium. The culture was allowed to incubate for two hours at $37^{o}C$ in 5% CO_2 atmosphere so that the macrophage could adhere to the glass coverslip. Following incubation, the coverslip was removed with forceps, rinsed twice with TC 199 medium and placed in another petri dish containing 10 ml of the appropriate amount of diluted latex particles in suspension. One ml of suspension was then taken and observed for engulf on latex particles with a Zeiss Phase Contrast Microscope.

XI. MEM Test for Malignant Disease

Three treatments were observed for the detection of malignant disease. The first treatment consisted of 2 ml guinea pig macrophages and 20 ml NaCl buffer. The second treatment consisted of 2 ml guinea pig macrophages, 20 ml NaCl and 10% Supernatant I.

Supernatant I consisted of 20 ml macrophages from rats which contained Walker 256 tumors. Macrophages were induced and isolated as discussed previously in this paper. Supernatant II was prepared the same as Supernatant I except the macrophage cells were taken from rats which were not induced with tumors.

XII. Assay for Glutamyl Transpeptidase

Peritoneal blood and liver macrophages were obtained from rats containing Walker 256 Carcinoma (tumors) and rats without Walker's 256 tumor. Three cell suspensions were set up for the control and tumor-induced rats. The first reaction tube contained .9 ml substrate and enzyme. The second contained .9 ml substrate and .9 ml buffer. The

third tube contained .9 ml enzyme and .9 ml buffer. Cells from these suspensions were then analyzed on a Spectrometer (Beckman 20) at 400 nm at one minute intervals for a total of 15 minutes. The macrophage was semi-purified by incubating for two hours at $37^{o}C$ in MEM on a glass slide. At the end of two hours the glass slide was washed twice with fresh MEM media and a rubber policeman's whistle was used to detach the cells from the glass slip. The cells were then centrifuged and resuspended in 10 ml of Tris HCl buffer (pH 9). This whole cell was used as the enzyme pre-paration.

The substrate was prepared so that 0.9 ml contains 5 micromoles L-glutamyl-p-nitroanilide, 10 micromoles $MgCl_2$, and 100 micromoles Tris HCl buffer (pH 9). To achieve a complete solution the substrate was heated to $55^{o}C$.

RESULTS AND DISCUSSION

Macrophage Culture Experiments

Stuart et al.,(1974) reported that macrophage cells cannot remain alive longer than 48 hours in cell culture. This physiological state makes them unfavorably to be used as candidates for space experiments. To study electrophoretic mobility of macrophage cells in space would require a cell viability greater than 30 days. An experiment was designed to increase the fetal calf serum content reported by Stuart et al., (1974) from 7% to 15%. It was found that a zero viability did not occur until the fourth day (Table 1). Therefore, the increase in the concentration of fetal calf serum resulted in the cells remaining alive two days longer. This could possibly be due to an increase in binding of cell surface and phagocytosis due to the serum. The results indicated that not only nutrients could be the influencing factor in the survival of macrophage cells in vitro, but also the accumulation of toxic cell products as observed by the rapid acidity of the culture media. The pH of the batch culture system changed from 7.2 to 6.2 within 72 hours. This demonstrated growth and production of organic acids. To increase cell viability, a culture system was designed to remove the accumulation of organic acids and other toxic cellular products. Table 2 reported the survival of guinea pig peritoneal macrophage cells in a continuous culture system. It can be noted that there was very slight change in pH from 7.2 to 7.4. An increase not a decrease was observed in the batch culture system. There was a 34% reduction in viable cells in

Table 1. Viabilities of Macrophages in Batch Culture

Time (hours)	Number of Macrophages*	% Viability
0	18×10^5	95
24	16.5×10^5	73
48	15.1×10^5	50
72	9.0×10^5	5
84	5.4×10^5	0

* The data represents an enumeration of the total cells present in the sample, both alive and dead.

Table 2.   The Survival of Macrophages In A Continuous Cultuvation System

Time (hours)	Number of Macrophages (Cells/ml)	% Viability	pH of Media
0	18×10^5	76	7.2
24	17.3×10^5	72	7.3
48	15.1×10^5	60	7.4
72	9.0×10^5	50	7.4

the continuous culture of the three days (Table 2) as compared to a 94% reduction in the batch culture (Table 1). This suggested that cells could remain viable longer if toxic products were removed as in a continuous system.

Figure 2 shows an illustration of the continuous culture system. The column was packed with glass beads to allow a surface for the macrophage to be attached. This instrumentation used in the experiment was capable of controlling the flow of fresh media at 1 ml/min. The slow growth rater of macrophages in submerged cultures could possibly be attributed to the reduction in the number of cells from 18 X 10^5 to 9 X 10^5 (Table 2). The lowest dilution rate that could be obtained was 1.2 hour^{-1} which was greater than the growth rate, thus resulting in loss of cells by washing out. An attempt to increase the growth rate of macrophage cells in the continuous culture system was performed by supplementing fresh media with serum protein isolated from the guinea pig used as the source of macrophage cells. The number of macrophage cells and pH remained constant over the three-day growing period at 15 X 10^5 and 7.0, respectively (Table 3). This suggested that a steady-state condition was obtained where the instantaneous growth rate of macrophage cells equals that of the dilution rate, resulting in no loss in cells by washing out. Guinea pig serum was used instead of fetal calf serum which was obtained from another animal species. In conclusion, the removal of old culture media and the use of protein isolated guinea pigs increased the survival of macrophage in vitro.

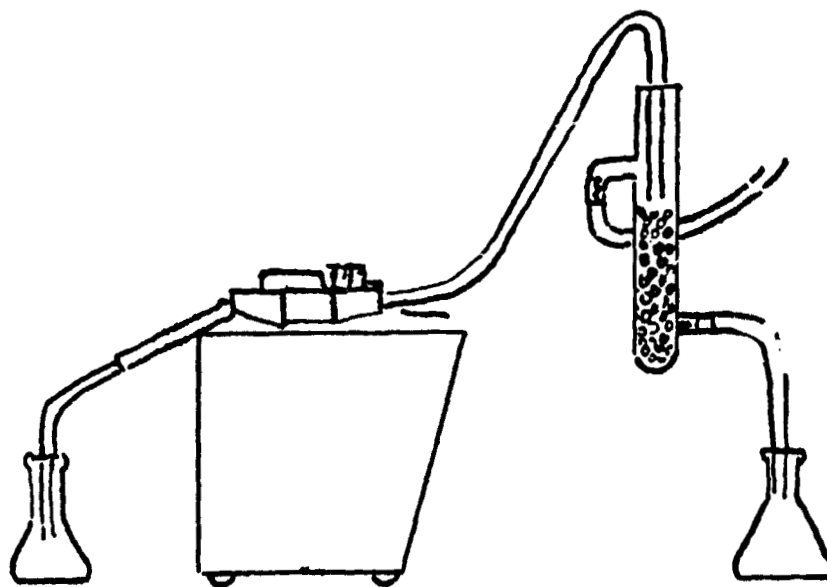Consultation with Dr. Linda Washington, Howard University, generated

Figure 2. Continuous culture system

Table 3.   The Survival of Macrophages In A Continuous Cultivation System
with Media Containing Guinea Pig Serum

Time (hours)	Number of Macrophages (Cells/ml)	% Viability	pH of Media
0	18×10^5	97	7.0
24	18×10^5	90	7.0
48	18×10^5	90	7.0
72	18×10^5	87	7.0

a method that yielded no percent viabilities of macrophages in batch culture (Table 4). The following modifications were made using this method.

(1) 4% Thioglycolate was used to induce macrophage instead of mineral oil.

(2) The culture media contained 20% fetal calf serum instead of 7 or 15%.

(3) Fresh media with serum was added every 24 hours.

As shown in Table 4, a constant cell concentration was obtained and no reduction in viability over a five-day growth period resulted. The constant cell concentration, in part, could be due to the attachment of cells to the flask surface and when the fresh media is added, any unattached cell will be removed. Secondly, the high viability could be attributed to a slight toxicity of mineral oil on macrophage cells. These cells engulf the mineral oil and oil droplets can be observed under a microscope. During cellular growth the oil could inhibit nutrient diffusion, movement and assimilation, and thus the cell is deprived of essential nutrients for its survival. By using Thioglycolate instead of mineral oil, this will not occur because these cells can catabolize Thioglycolate and utilize it as a carbon and sulfur source. Also, Thioglycolate will maintain a lower redox potential of the media which is more favorable for the growth of these cells.

Freezing experiments were initiated in an effort to increase the survival time and case of handling the macrophages for space studies. After six days of storage, $4^{0}C$ and ^{0}C, the macrophage cells were

Table 4. The Survival of Marcophages in Culture*

Time (hours)	Number of Macrophages (Cells/ml)	% Viability
0	18×10^5	100
24	18×10^5	100
48	18×10^5	100
72	18×10^5	100
84	18×10^5	100
96	18×10^5	100

* Macrophage cells were induced with 4% Thioglycolate and the culture media contained 20% Fetal Calf Serum.

still about 50% viable (Table 5). Of the two storage temperatures, $4^{o}C$ had the higher viability.

Viability was attributed to an increase in concentration of cryogenic protective agents (glycerol) from 7 to 15%. The decrease in the total number of cells of the sample stored at $4^{o}C$ could possibly indicate cell autolysis. The thawing process of the frozen sample $(-70^{o}C)$ appeared to cause cell rupture as indicated by the decrease in macrophage viability. To avoid cell rupture by ice formation during freezing, an experiment was designed to determine an optimal concentration of cryogenic protective agents.

The results of this experiment indicated that adding certain preservatives to the stored media increased the survival time of macrophages (Table 6). The data in Table 6 shows that glycerol initiated higher cell viability between 5% and 10% concentration as compared to Dimethyl Sulphoxide (DMSO). However, above 10% the influence of DMSO on the membrane surface prevented intracellular ice formation. If the cells were completely impermeable to an additive, they should shrink as a result of the osmotic withdrawal of water, and it should remain shrunken. On the other hand, if cells were completely permeable to an additive, they should shrink initially from loss of water and then expand back to their original volume as the additive permeates. DMSO is a rapidly permeating agent when exposed to cells, thus probably will be a more effective cytogenetic protective agent at higher concentration than glycerol. Glycerol is partially able to penetrate the cell as a result of diffusion. The glycerol protects the cells on a molar basis by reducing

Table 5.   The Survival of Macrophages Stored at $4^{O}C$ and $-70^{O}C$

Time (Days)	Stored at $4^{O}C$ # of Macrophages (Cells/ml)	% Viability	Stored at $-70^{O}C$ # of Macrophages (Cells/ml)	% Viability
0	18×10^{5}	75	22×10^{5}	78
6	31×10^{5}	55	22×10^{5}	48

Table 6.   The Viability of Macrophage Cells Stored in DMSO and Glycerol

DMSO Concentra- tions % V/V	% Viability	Glycerine Con- centrations % V/V	% Viability
1	—*	1	—
2	—	2	—
5	13	5	27
10	50	10	32
15	33	15	21

* The (—) represents sample loss due to breakage.

the electrolyte concentration in the residual unfrozen solution in and around a cell at any given temperature.

The total mole fraction of a glycerol solution at a constant pressure is determined by temperature alone. If all the solutes are electrolytes, then the required mole fraction will consist entirely of electrolytes. But if a non-electrolyte such as glycerol is present, the concentration of electrolytes will be reduced and the extent of reduction at a given temperature will be approximately proportional to the osmolar ratio of glycerol to electrolyte in the initial suspension. The data in Figure 3 shows that the influence of Glycerol concentration when supplemented in Media 199 on the viability of macrophages stored at $-196^{\circ}C$. Cells frozen in media containing 5% glycerol showed a slower rate of death. The reverse in phenomenon showing macrophage cells with 10 and 15% glycerol concentration under a slow cooling rate, extensively reduced the required mole fraction of salt electrolyte to a lethal level. Another interpretation is that glycerol penetrated cells and increased the cell volume causing rupture of the cell membrane. There is also a difference in mobility of cells stored in DMSO as compared to glycerol (Table 7). The mobility readings at 5% glycerol were very close to the mobility rate for non-treated macrophage cells. At concentrations in greater volume than 10%, the mobility rates were much higher. This increase could possibly be due to readings made on dead cells. The non-viable cells were found to have a higher electrophoretic mobility.

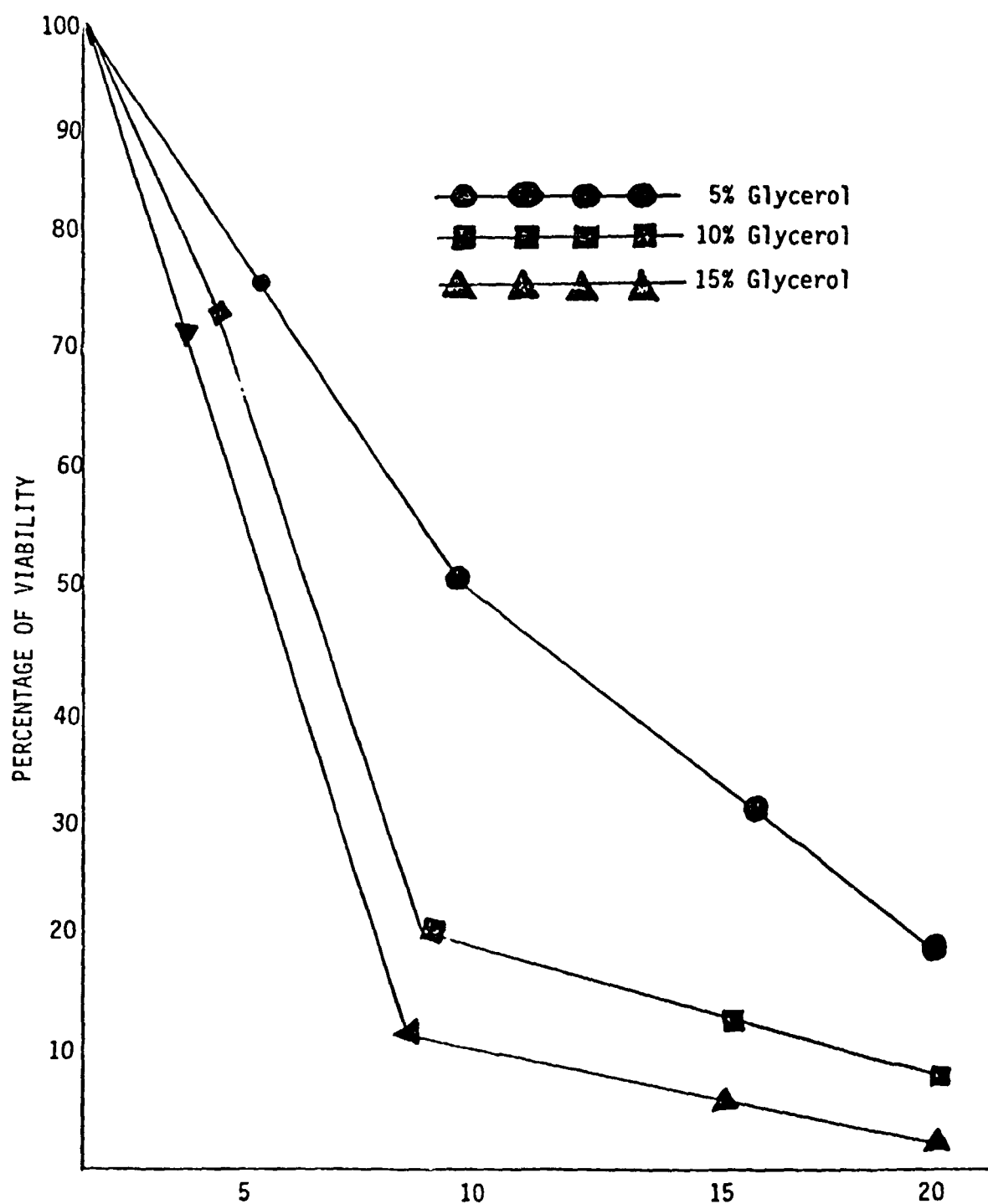The storage of cells under liquid nitrogen did not alter the

Figure 3. Percentage of Viable Macrophage Cells When Exposed in
Different Concentrations of Glycerol During Freezing

Table 7. The Electrophoretic Mobilities of Macrophages in DMSO and Glycerol

DMSO Concentrations % V/V	Electrophoretic Mobilities Mu/Sec/V/Cm	Glycerol Concentrations	Electrophoretic Mobilities Mu/Sec/V/Cm
1	0.724 ± 0.01	1	0.596 ± 0.007
2	0.302 ± 0.03	2	0.646 ± 0.11
5	1.42 ± 0.05	5	1.08 ± 0.015
10	0.875 ± 0.015	10	1.09 ± 0.098
15		15	1.32 ± 0.098

* These cells were stored at -80°C for five days before readings were made.

activities of the cells as shown in Table 8. However, the cell viability
was not maintained. The unstable cell viability in this experiment can
be attributed to cell shock resulting from a change in temperature during
storage. The temperature changed from $-196^{\circ}C$ to $-70^{\circ}C$.

The optimal freezing rate of the macrophage cells using 7% glycerol
as the protective agent was $3^{\circ}C/min$. This is not necessarily the cooling
rate that yields maximum survival. Mazur et al., (1970) reported that op-
timal rates can differ by at least a factor of 2000 in different cells.
Furthermore, even in a given type of cell the optimum can vary with con-
ditions of freezing rate between $3^{\circ}C/min$ and rapid freezing by submerg-
ing in liquid nitrogen. These results suggest that two factors affect
cell viability: (1) cells cooled more slowly are injured chiefly by
solute concentration or dehydration, and (2) cells cooled more rapidly
are injured chiefly by intracellular freezing and by recrystallization
during thawing. The fact that glycerol can confer protection during slow
cooling suggests that the surface of the cell membrane is especially suscep-
tible to damage by freezing. The results of freezing experiments were
encouraging and could possibly be an economical and simple means of
storing the cells, provided that higher viability and electrophoretic
characteristics of the cells could be maintained.

Over the years, several strains of rats and mice have been obtained
by continual in-breeding, and certain of these strains are prone to a ·
high incidence of a particular cancer. Figure 4 shows a mouse with two
breast cancers from a strain that shows a very high incidence of these
two tumors. In this case the cancer is caused by surgical transplant.

Table 8. Effects of Temperature Rate of Freezing Activities of Macro-
phage Cells

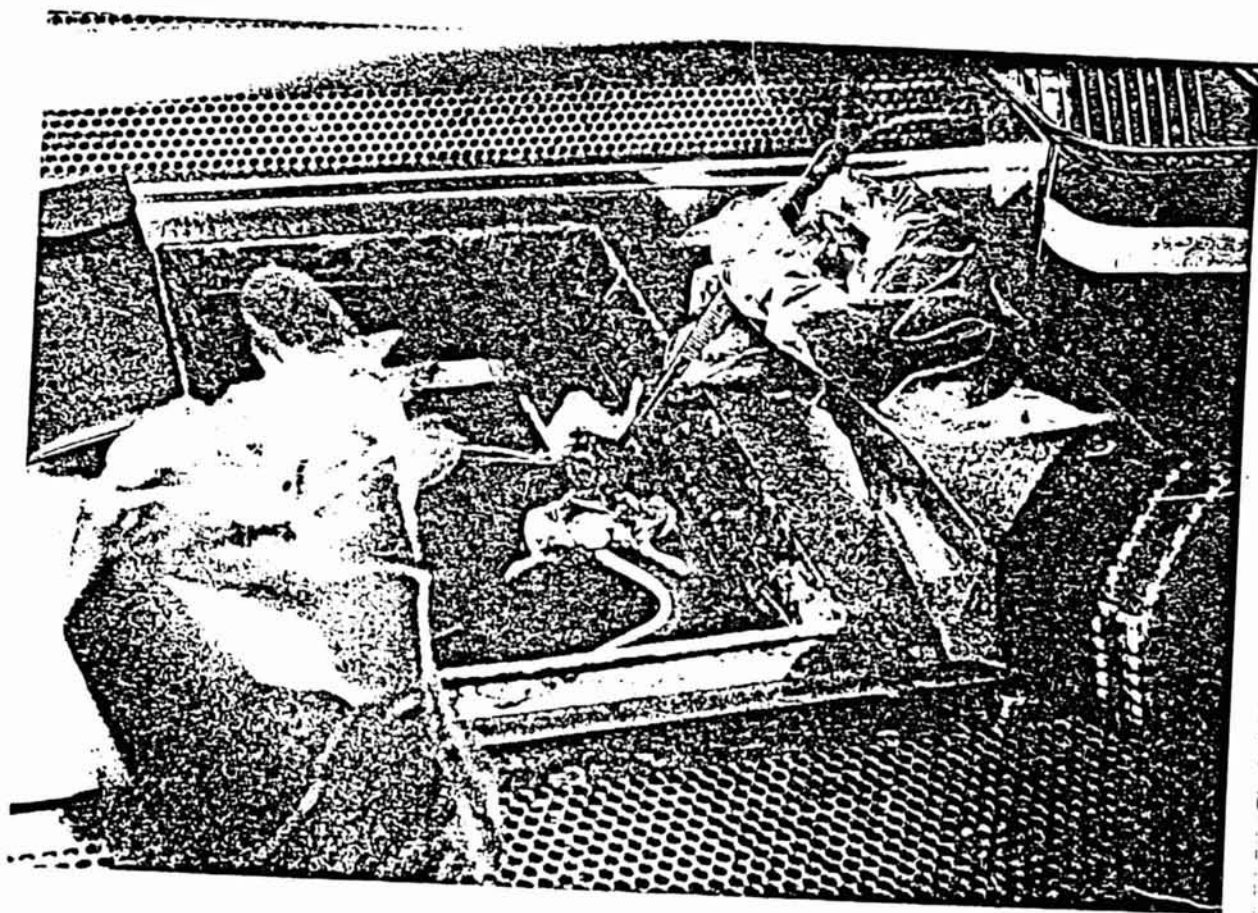| Freezing Rate (C/min)	% Viability	% Phagocytosis
0	87	73
1	40	38
3	61	55

Figure 4. Mammary Tumor Cells

In many cases, the cancer is caused by a virus which is transmitted from generation to generation in the milk of the mother.

Alternatively, cancers can be made to arise in animals by a number of methods such as the application of chemicals or the administration of viruses. However, the most favored method, because it requires less space and a smaller number of animals, is to obtain the tumor by transplantation or by injecting a subcutaneous cell mixture. The tumors in this series (a malignant melanoma and a Walker 256) are locally invasive and tend to infiltrate striated muscle and fatty tissue (Figure 4). In tumors exceeding 5 mm in diameter necrosis of central area is observed, surrounded by a rim of nonnectotic tumor tissue at the periphery. Transplanted tumors grow locally at the site of implantation and metastatic spreading to lymph nodes or organs has not been observed.

Macrophage Membrane Enzyme Activity

It has been recently reported that gamma-glutamyl transpeptidase activity is higher in tumor-containing individuals than normal population. This enzyme is associated with the cell membrane and activity could possibly be involved in the electronegative change different in macrophage cells. An experiment was designed to measure the activity of this enzyme in rats induced with Walker 256 tumor and mammary tumor. A significant amount of gamma-glutamyl transpeptidase activity was not noticeable in these animals (Figure 5). The control group, 4% Thioglycolate, initiated macrophage cells but very little cells were isolated from peritoneal of rats containing mammary carcinoma. Low concentrations of cells may be one explanation for gamma-glutamyl transpeptidase activity being zero.
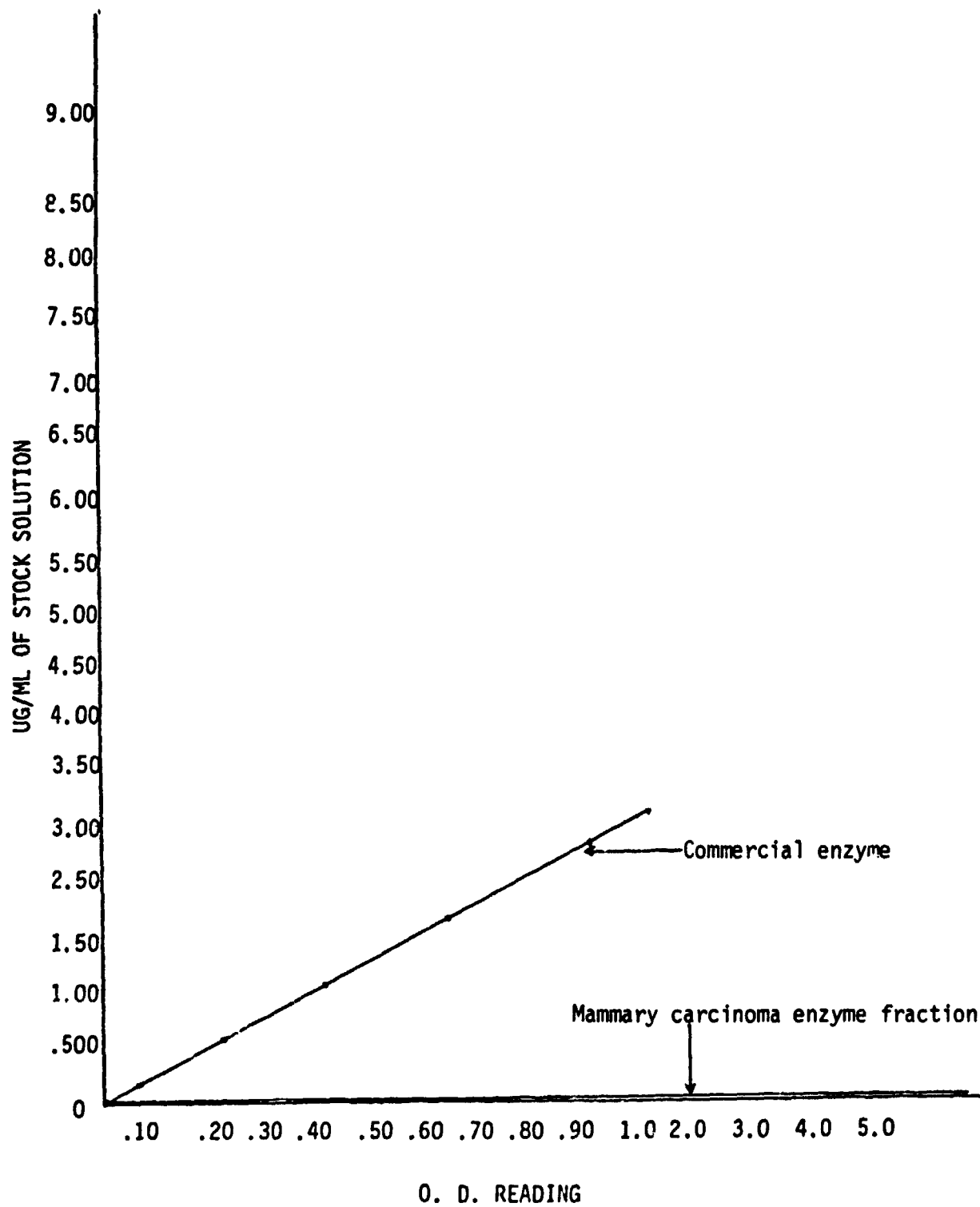
Figure 5. The amount of gamma-glutamyl transpeptidase activity

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